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African Swine Fever Virus Georgia Isolate Harboring Deletions of MGF360 and MGF505 Genes Is Attenuated in Swine and Confers Protection against Challenge with Virulent Parental Virus

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ABSTRACT

African swine fever virus (ASFV) is the etiological agent of a contagious and often lethal disease of domestic pigs that has significant economic consequences for the swine industry. The control of African swine fever (ASF) has been hampered by the unavailability of vaccines. Experimental vaccines have been developed using genetically modified live attenuated ASFVs where viral genes involved in virus virulence were removed from the genome. Multigene family 360 (MGF360) and MGF505 represent a group of genes sharing partial sequence and structural identities that have been connected with ASFV host range specificity, blocking of the host innate response, and virus virulence. Here we report the construction of a recombinant virus (ASFV-G-ΔMGF) derived from the highly virulent ASFV Georgia 2007 isolate (ASFV-G) by specifically deleting six genes belonging to MGF360 or MGF505: MGF505-1R, MGF360-12L, MGF360-13L, MGF360-14L, MGF505-2R, and MGF505-3R. ASFV-G-ΔMGF replicates as efficiently in primary swine macrophage cell cultures as the parental virus. *In vivo*, ASFV-G-ΔMGF is completely attenuated in swine, since pigs inoculated intramuscularly (i.m.) with either 10^2 or 10^4 50% hemadsorbing doses (HAD_{50}) remained healthy, without signs of the disease. Importantly, when these animals were subsequently exposed to highly virulent parental ASFV-G, no signs of the disease were observed, although a proportion of these animals harbored the challenge virus. This is the first report demonstrating the role of MGF genes acting as independent determinants of ASFV virulence. Additionally, ASFV-G-ΔMGF is the first experimental vaccine reported to induce protection in pigs challenged with highly virulent and epidemiologically relevant ASFV-G.

IMPORTANCE

The main problem for controlling ASF is the lack of vaccines. Studies focusing on understanding ASFV virulence led to the production of genetically modified recombinant viruses that, while attenuated, are able to confer protection in pigs challenged with homologous viruses. Here we have produced an attenuated recombinant ASFV derived from highly virulent ASFV strain Georgia (ASFV-G) lacking only six of the multigene family 360 (MGF360) and MGF505 genes (ASFV-G-ΔMGF). It is demonstrated, by first time, that deleting specific MGF genes alone can completely attenuate a highly virulent field ASFV isolate. Recombinant virus ASFV-G-ΔMGF effectively confers protection in pigs against challenge with ASFV-G when delivered once via the intramuscular (i.m.) route. The protection against ASFV-G is highly effective by 28 days postvaccination. This is the first report of an experimental vaccine that induces solid protection against virulent ASFV-G.

African swine fever (ASF) is a contagious viral disease of swine caused by ASF virus (ASFV), a large enveloped virus containing a double-stranded DNA genome of approximately 180 to 190 kbp (1). ASF causes a spectrum of disease manifestations, from highly lethal to subclinical, depending on host characteristics and the virus strain (2). Virulent ASFV infections in domestic pigs are fatal and characterized by fever, hemorrhages, ataxia, and severe depression.

Currently, ASF is endemic in several sub-Saharan African countries. In Europe, the disease is endemic in Sardinia (Italy), and outbreaks have been recorded in the Caucasus region since 2007, affecting Georgia, Armenia, Azerbaijan, and Russia, and, more recently, in Ukraine, Belarus, Lithuania, Latvia, and Poland, threatening to disseminate into neighboring western European countries (3).

There is no vaccine available for ASF, and outbreaks are usually controlled by animal quarantine and elimination of the affected animals. Experimental vaccines based on the use of different inactivated virus preparations have failed to induce protective immunity (4–6). Protective immunity against reinfection with homologous viruses and (rarely) against reinfection with heterologous viruses does develop in pigs surviving viral infection (7, 8). Pigs immunized with live attenuated ASF viruses containing engi-

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neered individual deletions of ASFV virulence-associated genes (genes *UK*, *DP69R*, *23-NL*, *TK*, and *9GL*) were protected when challenged with homologous virulent parental virus (9–12). Thus far, those studies have provided the only experimental evidence describing the rational development of an effective live attenuated virus against ASFV.

The ASFV genome contains genes constituting several distinct multigene families (MGF), originally characterized as genes present as repetitive sequences in terminal genomic regions and named to reflect the average lengths of the predicted gene product (13, 14). Two of these families, MGF360 and MGF505, are present in the highly variable left-terminal genomic region and encode products sharing structural similarities (13–18).

Some cell culture-adapted ASFV isolates (such as the Vero cell-adapted Badajoz71 or Georgia07 isolates) contain gaps in the variable left-terminal region of their genomes harboring deletions that encompass several genes of both MGF360 and MGF505 (15, 19–22). Usually, adaptation to replication in these cell lines coincides with attenuation of these isolates in swine (8, 22–25). However, there is no conclusive evidence supporting the idea of a cause-effect mechanism operating between the deletion of specific MGF genes, cell culture adaptation, and virus attenuation in swine.

Investigating the MGF360 and MGF505 regions may elucidate their roles in the ASFV replication cycle and/or their contributions to virulence. For instance, deletion of MGF genes in the Vero cell-adapted ASFV BA71 isolate reduced the ability of the virus to replicate in swine macrophages (26, 27), and partial reconstitution of the gap observed in the genome, located within the variable left-terminal region, led to the virus regaining the ability to replicate in swine macrophages (26). In addition, it has been observed that genes within the MGF360 and MGF505 regions are important for enabling virus replication in tick cells (28). It has also been reported that MGF360 and MGF505 genes are involved in the inhibition of interferon (IFN) production (29). MGF360 and MGF505 genes have also been implicated in virus virulence; deletion of genes within these MGFs contributed to attenuation of the virulent recombinant Malawi Lil-20/1ΔNL strain (30), whose NL virulence-associated viral gene had already been deleted (31).

Here, we report the construction of a recombinant virus derived from the highly virulent ASFV Georgia 2007 (ASFV-G) isolate by specifically deleting six genes of the ASFV MGF360 and MGF505 families (ASFV-G-ΔMGF). It was shown that ASFV-G-ΔMGF replicates in primary swine macrophage cell cultures as efficiently as the parental virus. *In vivo*, ASFV-G-ΔMGF administered intramuscularly (i.m.) to swine at doses as high as 10^4 50% hemadsorbing doses (HAD_{50}) is completely attenuated. Importantly, animals infected i.m. with 10^2 or 10^4 HAD_{50} of ASFV-G-ΔMGF were protected against clinical disease when challenged at 28 days postinfection (dpi) with highly virulent parental ASFV-G. This constitutes the first report demonstrating that deletion of specific members of MGF360 and MGF505 is sufficient to produce a complete attenuation of a virulent field isolate and that the resulting recombinant virus is able to induce an effective protective response against challenge with the parental virus, ASFV-G.

MATERIALS AND METHODS

Cell cultures and viruses. Primary swine macrophage cell cultures were prepared from defibrinated swine blood as previously described by Zsak et al. (11). Briefly, heparin-treated swine blood was incubated at 37°C for 1 h to allow sedimentation of the erythrocyte fraction. Mononuclear leukocytes were separated by flotation over a Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient (specific gravity, 1.079). The monocyte/macrophage cell fraction was cultured in plastic Primaria tissue culture flasks (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) containing macrophage media, composed of RPMI 1640 medium (Life Technologies, Grand Island, NY) with 30% L929 supernatant and 20% heat-inactivated fetal bovine serum (HI-FBS; Thermo Scientific, Waltham, MA), for 48 h at 37°C under 5% CO_2. Adherent cells were detached from the plastic by using 10 mM EDTA–phosphate-buffered saline (PBS) and were then reseeded into Primaria T25 6- or 96-well dishes at a density of 5×10^6 cells per ml for use in assays 24 h later.

ASFV Georgia (ASFV-G) was a field isolate kindly provided by N. Vepkhvadze of the Laboratory of the Ministry of Agriculture (LMA) in Tbilisi, Republic of Georgia (22).

Comparisons of growth curves of ASFV-G and ASFV-G-ΔMGF viruses were performed in primary swine macrophage cell cultures. Preformed monolayers were prepared in 24-well plates and infected at a multiplicity of infection (MOI) of 0.1 or 0.01 (based on the HAD_{50} previously determined in primary swine macrophage cell cultures). After 1 h of adsorption at 37°C under 5% CO_2, the inoculum was removed and the cells were rinsed two times with PBS. The monolayers were then rinsed with macrophage media and incubated for 2, 24, 48, 72, and 96 h at 37°C under 5% CO_2. At appropriate times postinfection, the cells were frozen at −70°C or lower and the thawed lysates were used to determine titers by HAD_{50}/ml in primary swine macrophage cell cultures. All samples were run simultaneously to avoid interassay variability.

Virus titration was performed on primary swine macrophage cell cultures in 96-well plates. Virus dilutions and cultures were performed using macrophage medium. The presence of virus was assessed by hemadsorption (HA), and virus titers were calculated by the Reed and Muench method (32).

Construction of the recombinant ASFV-G-ΔMGF strain. Recombinant ASFVs were generated by homologous recombination between the parental ASFV genome and a recombination transfer vector following infection and transfection of swine macrophage cell cultures (11, 33). The recombinant transfer vector (p72GUSΔMGF) contained flanking genomic regions, which included the amino terminus of the MGF505-1R gene mapping to the left (the left arm is located between genomic positions 24566 and 27928) and the carboxyl end of the MGF505-3R gene mapping to the right (the right arm is located between genomic positions 35487 and 36515) and a reporter gene cassette containing the β-glucuronidase (β-GUS) gene with the ASFV p72 late gene promoter (11). This construction created a 7,558-nucleotide deletion in the left variable region of ASFV-G (between nucleotide positions 27928 and 35487) (Fig. 1). Recombinant transfer vector p72GUSΔMGF was obtained by DNA synthesis (Epoch Life Sciences, Sugar Land, TX, USA). Macrophage cell cultures were infected with ASFV-G and transfected with p72GUSΔMGF. Recombinant viruses representing independent primary plaques were purified to homogeneity by successive rounds of plaque assay purification.

PCR. The purity of ASFV-G-ΔMGF in the virus stock was assessed by PCR. Detection of the MGF350/MGF505 genes was performed using the following pair of primers: forward, 5′-GAGGATGATTTGCCCTTCACT CA-3′; reverse, 5′-CGCCACTAGTAAACATTGTTCTATCT-3′. These primers amplified a 422-bp fragment of the open reading frame (ORF) MGF505-1R gene. Detection of the β-GUS gene was performed using the following pair of primers: forward, 5′-GACGGCCTGTGGGCATT-3′; reverse, 5′-GCGATGGATTCCGGCAT-3′. Detection of the *p72* (*B646L*) gene was performed using the following pair of primers: forward, 5′-GT CTTATTGCTAACGATGGGAAG3′; reverse, 5′-CCAAAGGTAAGCTT GTTTCCCAA-3′.

Sequencing of PCR products. PCR products were sequenced using the dideoxynucleotide chain termination method (34). Sequencing reactions were prepared with a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on

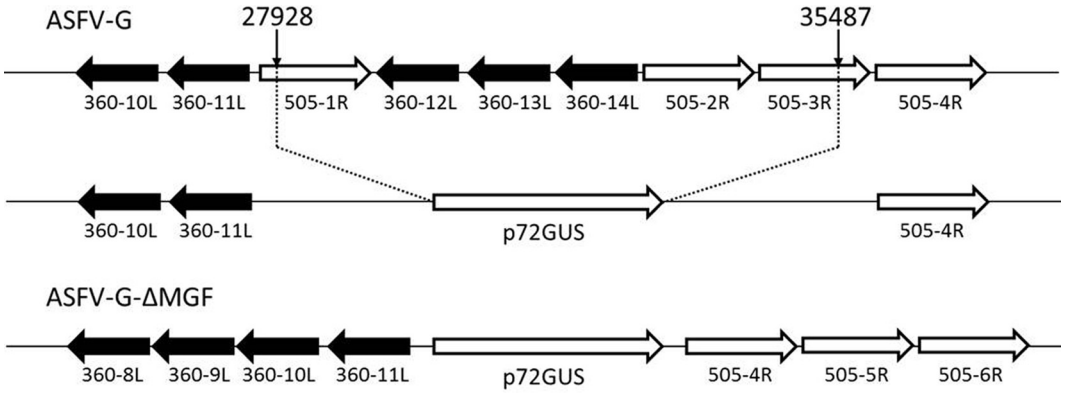

FIG 1 Schematic representation of the MGF360 and MGF505 gene region deleted in ASFV-G-ΔMGF and replaced with the p72GUS reporter gene cassette. Nucleotide positions indicating the boundaries of the deletion relative to the ASFV-G genome are indicated.

a Prism 3730xl automated DNA sequencer (Applied Biosystems). Sequence data were assembled with the Phrap software program (http://www.phrap.org), with confirmatory assemblies performed using CAP3 (35). The final DNA consensus sequence represented an average 5-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (Tom Hall, Ibis Biosciences, Carlsbad, CA).

Next-generation sequencing (NGS) of ASFV genomes. ASFV DNA was extracted from infected cells and quantified as described earlier (22). Full-length sequencing of the virus genome was performed as described elsewhere (22). Briefly, 1 μg of virus DNA was enzymatically sheared and the resulting fragmented DNA size distribution was assessed. Adapters and library barcodes were ligated to the fragmented DNA. The appropriate size range of the adapter-ligated library was collected using the Pippin Prep system (Sage Science) followed by normalization of the library concentration. The DNA library was then clonally amplified onto Ion sphere particles (ISPs) and enriched. Enriched template ISPs were prepared and loaded onto Ion chips for sequencing. Sequence analysis was performed using Galaxy (https://usegalaxy.org/) and CLC Genomics Workbench (CLCBio).

Animal experiments. Animal experiments were performed under biosafety level 3 conditions in the animal facilities at the Plum Island Animal Disease Center (PIADC) following a protocol approved by the Institutional Animal Care and Use Committee.

ASFV-G-ΔMGF was assessed for its virulence phenotype relative to that of the parental ASFV-G virus using 80-to-90-pound commercial breed swine. Five pigs were inoculated intramuscularly (i.m.) with either 10^2 or 10^4 HAD_{50} of ASFV-G-ΔMGF or ASFV-G. Clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea, and cough) and changes in body temperature were recorded daily throughout the experiment.

To assess the protective effect of ASFV-G-ΔMGF, at 28 days postinfection, ASFV-G-ΔMGF-infected animals were i.m. challenged with 10^3 HAD_{50} of highly virulent parental ASFV-G. Clinical signs (as described above) and changes in body temperature were recorded daily throughout the experiment.

Detection of anti-ASFV antibodies. Anti-ASFV antibodies in sera of infected animals were quantified using an assay developed in-house. Vero cells were infected (MOI = 0.1) with the ASFV Vero cell-adapted Georgia strain (22) in 96-well plates. Two-fold dilutions of the sera were incubated for 1 h at 37°C in the 96-well ASFV-infected cell monolayer. After washing was performed, the presence of anti-ASFV antibodies was detected by using a commercial anti-swine peroxidase-labeled mouse immunoglobulin and a peroxidase substrate (Vector Laboratories, Burlingame, CA). Titers were expressed as the $\log_{10}$ of the inverse of the highest serum dilution showing a reaction with the infected cells.

RESULTS

Amino acid identity of putative proteins encoded by ORFs MGF360-12L, -13L, and -14L and MGF505-1R, -2R, and -3R. Putative proteins encoded by ORFs MGF360-12L, -13L, and -14L and MGF505-1R, -2R, and -3R of ASFV-G were compared with those of ASFV isolates of different geographical and temporal origins (Table 1 and Fig. 2). Despite the differences, a high degree of conservancy was observed among these ORFs. Identity of 84% and above was observed among these ORFs, with the lowest level of identity observed with the Kenya isolate obtained in 1950. These field isolates are considered virulent for domestic pigs, with the exception of the Mkuzi, Warmbaths, and Warthog field isolates, whose pathogenicity is unknown. In contrast, alignment of the left variable genome region of nonvirulent ASFV field isolates OURT88/3 and NHV with that of ASFV-G showed deletions encompassing different members of MGF360 and MGF505 ORFs, including MGF360-12L, -13L, and -14L and MGF505-1R, -2R, and -3R, indicating a possible role of these gene families in the pathogenicity of ASFV.

Development of the ASFV-G-ΔMGF deletion mutant. ASFV-G-ΔMGF was constructed from the highly pathogenic ASFV Georgia 2007 isolate (ASFV-G). A 7,558-bp region (between nucleotide positions 27928 and 35487) was deleted from the ASFV-G virus and replaced with a cassette containing the p72GUS reporter gene by homologous recombination (see Materials and Methods). The resulting recombinant virus harbors a deletion encompassing the carboxyl-terminal half of the MGF505-1R gene and complete deletion of the MGF360-12L, -13L, and -14L and MGF505-2R genes along with a deletion of the amino-terminal half of the MGF505-3R gene (Fig. 1). The recombinant virus was obtained after eight successive plaque purification events on monolayers of primary swine macrophage cell cultures. The virus population obtained from the last round of plaque purification was amplified in primary swine macrophage cell cultures to obtain a virus stock.

To ensure the absence of parental ASFV-G, virus DNA was extracted from the virus stock and analyzed by PCR using primers targeting genes p72 (*B646L*), MGF, and β-GUS. Only amplicons for the p72 (*B646L*) and β-GUS genes were detected in DNA extracted from the virus stock; no amplicons were generated with primers targeting the MGF505-1R gene (Fig. 3), indicating the lack of contamination of the ASFV-G-ΔMGF stock with ASFV-G.

TABLE 1 Amino acid identities of putative proteins encoded by ORFs MGF360-12L, -13L, and -14L and MGF505-1R, -2R, and -3R of different ASFV isolates relative to those of ASFV-G

Isolate	GenBank accession no.	Geographical origin	Host	Yr of isolation	Virulence	MGF360 ORF identity[a]			MGF505 ORF identity[b]		
						12L	13L	14L	1R	2R	3R
Tengani 62	AY261364	Malawi	Pig	1962	High	0.994	0.951	0.966	0.979	0.952	0.964
Mkuzi	AY261362	South Africa	Tick	1979	Unknown	0.957	0.960	0.988	0.915	0.988	0.935
Warmbaths	AY261365	South Africa	Tick	1987	Unknown	0.962	0.929	0.932	0.907	0.948	0.953
Benin 97/1	AM712239	Benin	Pig	1997	High	0.928	0.957	0.988	0.902	0.990	0.925
E75	FN557520	Spain	Pig	1975	High	0.940[a]	0.957	0.988	0.902	0.990[b]	0.925
L60	KM262844	Portugal	Pig	1960	High	0.934	0.957	0.988	0.902	0.990	0.925
Warthog	AY261366	Namibia	Warthog	1980	Unknown	0.962	0.937	0.966	0.896	0.933	0.964
Pretorisuskop/96/4	AY261363	South Africa	Tick	1996	High	0.965	0.923	0.960	0.883	0.944	0.957
Malawi Lil-20/1	AY261361	Malawi	Tick	1983	High	0.894	0.870	0.896	0.892	0.899	0.864
Kenya 1950	AY261360	Kenya	Pig	1950	High	0.866	0.841	0.899	0.862	0.861	0.834

[a] Identities of 117 carboxyl-terminal amino acids. E75 harbors a truncated form of the MGF360-12L ORF, lacking 233 residues at the amino-terminal end.
[b] Identities of 331 amino-terminal amino acids. E75 harbors a truncated form of the MGF505-2R ORF, lacking 169 residues at the carboxyl-terminal end.

Analysis of the ASFV-G-ΔMGF genome sequence relative to the parental ASFV-G genome sequence. To evaluate the accuracy of the genetic modification and the integrity of the genome of the recombinant virus, full-genome sequences of the ASFV-G-ΔMGF and parental ASFV-G strains were obtained using NGS and the Ion Torrent Personal Genome Machine (PGM) and compared. A full-length genome comparison between parental ASFV-G and ASFV Georgia 2007/1 (GenBank accession FR682468) (3) demonstrated the following results: the presence of two nucleotide insertions (a T nucleotide insertion and an A nucleotide insertion at nucleotide positions 430 and 441, respectively) in a noncoding segment at the 5′ of the virus genome; a double-frameshift mutation (a TT deletion and a T insertion at nucleotide positions 1602 and 1620, respectively) affecting the MGF360-1L gene; a silent mutation (at nucleotide position 97321) in ORF *B438L*; a residue substitution (Ala to Pro) at residue position 85 in ORF *E199L*; and a nucleotide insertion (at nucleotide position 183303) in a noncoding segment at the 3′ side of the genome (22). The full-length

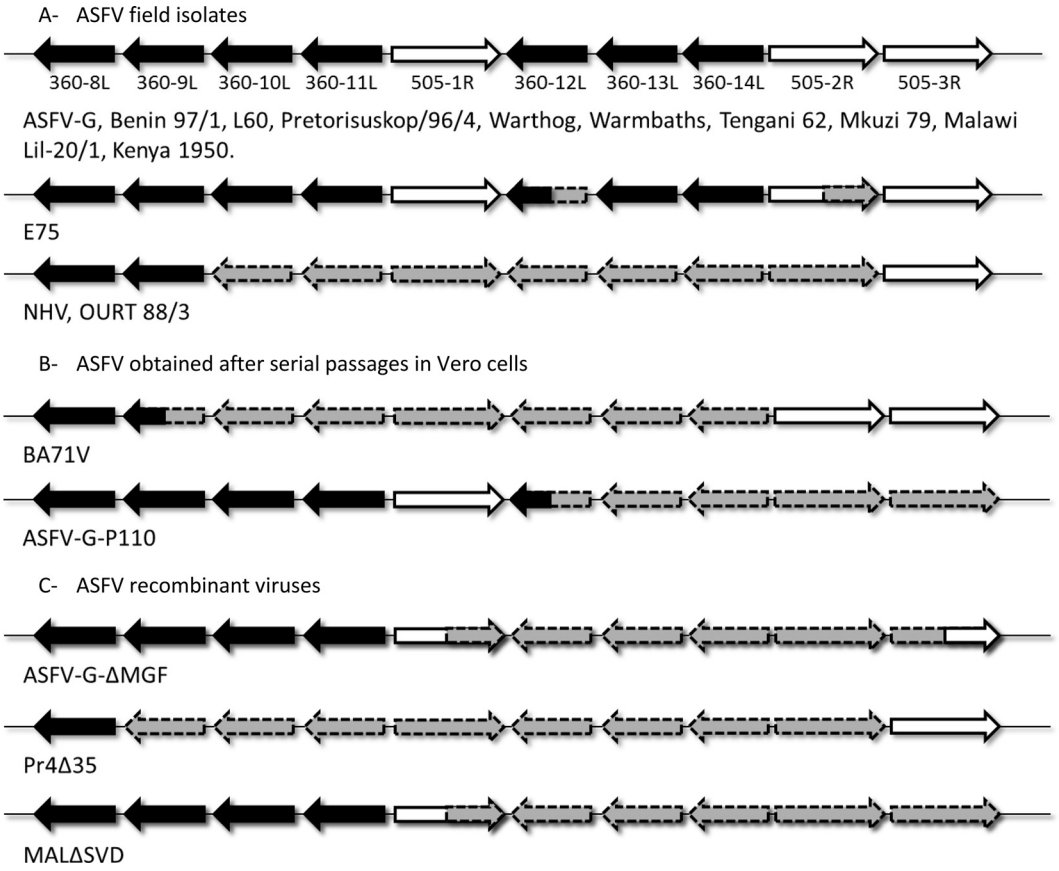

FIG 2 Schematic representation of the MGF360 and MGF505 gene region deleted in ASFV-G-ΔMGF compared with naturally occurring viruses (A), cell-passaged viruses (B), and recombinant viruses (C). Gray shaded and dotted arrows represent partial and total gene deletions.

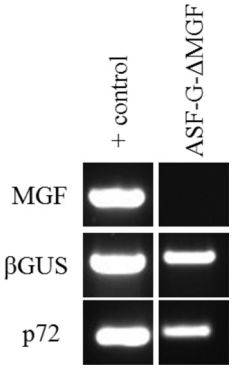

FIG 3 PCR analysis of ASFV-G-ΔMGF stocks using specific primers targeting MGF360, MGF505, p72 (*B646L*), or β-GUS genes. + control (positive control), ASFV-G DNA for detection of MGF360 and MGF505 and p72 genes and p72GUSΔMGF recombinant plasmid for β-GUS gene detection.

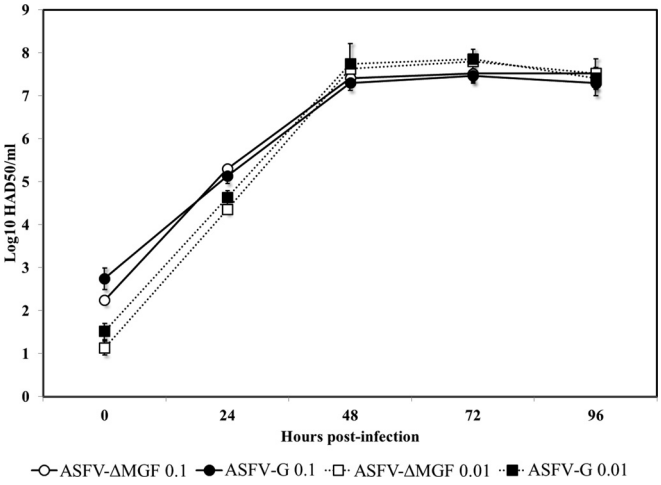

FIG 4 *In vitro* growth kinetics of ASFV-G-ΔMGF and parental ASFV-G. Primary swine macrophage cell cultures were infected (MOI = 0.1 or 0.01) with either ASFV-G-ΔMGF or parental ASFV-G viruses, and yields of virus titrated in primary swine macrophage cell cultures were obtained at the indicated times postinfection. Data represent means and standard deviations of the results from two independent experiments. Sensitivity of virus detection, $\geq \log_{10}$ 1.8 HAD_{50}/ml.

genome comparison between ASFV-G-ΔMGF and parental ASFV-G revealed a deletion of nucleotides (7,558 bp) corresponding to the introduced modification. Additionally, the consensus sequence of the ASFV-G-ΔMGF genome showed an insertion of 2,324 nucleotides corresponding to the p72GUS cassette sequence. Besides the insertion of the p72GUS cassette, no additional differences were observed between the ASFV-G-ΔMGF and ASFV-G genomes, confirming that the ASFV-G-ΔMGF virus did not accumulate mutations during the process of homologous recombination and plaque purification.

Replication of ASFV-G-ΔMGF in primary swine macrophages. MGF genes located in the left variable region of the ASFV genome have been described to be involved in ASFV replication in swine macrophages (22, 26, 27). The *in vitro* growth characteristics of ASFV-G-ΔMGF were evaluated in primary swine macrophage cell cultures, the primary cell targeted by ASFV during infection in swine, and compared to those of the parental ASFV-G strain in a multistep growth curve analysis. Cell cultures were infected with these viruses at an MOI of either 0.1 or 0.01, and samples were collected at 2, 24, 48, 72, and 96 h postinfection (hpi). ASFV-G-ΔMGF displayed a growth kinetic similar to that of the parental ASFV-G virus (Fig. 4). Therefore, deletion of MGF360 and MGF505 genes in ASFV-G-ΔMGF does not significantly affect the ability of the virus to replicate in primary swine macrophage cultures.

Assessment of ASFV-G-ΔMGF virulence in swine. In order to evaluate the effect of the deletion of MGF360 and MGF505 genes on ASFV-G-ΔMGF virulence, four groups of 80-to-90-pound pigs were i.m. inoculated with 10^2 or 10^4 HAD_{50} of either ASFV-G-ΔMGF ($n = 10$) or ASFV-G ($n = 5$). As expected, animals infected with 10^4 HAD_{50} of ASFV-G exhibited increased body temperature (>104°F) by 3 to 4 days postinfection followed by the appearance of clinical signs associated with the disease, including anorexia, depression, purple skin discoloration, staggering gait, and diarrhea (Table 2). Signs of the disease increased progressively over time, and animals either died or were euthanized *in extremis* by 7 to 8 days postinfection. Animals infected with 10^2 HAD_{50} of ASFV-G presented with similar disease, with the difference that the onset of clinical signs and time of death were delayed 3 to 4 days relative to the results seen with animals infected with 10^4 HAD_{50}. Conversely, pigs inoculated via the i.m. route with 10^2 or 10^4 HAD_{50} of mutant virus ASFV-G-ΔMGF did not present any signs of clinical disease during the entire observation period (21 days). Therefore, deletion of MGF360-12L, -13L, and -14L and MGF505-1R, -2R, and -3R completely attenuated highly virulent ASFV-G.

Viremia in experimentally inoculated animals was quantified at different days postinfection in swine macrophage cell cultures. As expected, animals inoculated with 10^2 or 10^4 HAD_{50} of virulent parental ASFV-G had very high virus titers in blood until the day of their death (Fig. 5). In both groups, viremia titers reached values as high as 10^7 to 10^8 HAD_{50}/ml by the time of death. Conversely, animals inoculated with 10^2 or 10^4 HAD_{50} of mutant ASFV-G-ΔMGF had relatively low virus titers in blood compared with those of the ASFV-G-inoculated animals. Animals inoculated with 10^2 HAD_{50} of mutant ASFV-G-ΔMGF presented a heterogeneous pattern of virus titers in blood. While three animals presented detectable levels (our test sensitivity was $\geq 10^{1.8}$ HAD_{50}/ml), six of them exhibited intermediate titer values (10^2 to 10^4 HAD_{50}/ml) and only one had viremia titers reaching 10^5 HAD_{50}/ml (Fig. 6A). Almost all animals showed negative results ($\leq 10^{1.8}$ HAD_{50}/ml) by the day of challenge (28 dpi). Animals inoculated with 10^4 HAD_{50}/ml of ASFV-G-ΔMGF presented patterns of viremia that were also heterogeneous, although, in general, they had higher viremia values than those inoculated with 10^2 HAD_{50}. In this group, 4 of 10 animals presented with viremia values greater than 10^4 HAD_{50}/ml at least at some sample points, while the other 6 animals exhibited viremias with maximum virus titers rarely reaching 10^3 to 10^4 HAD_{50}/ml (Fig. 6B). At the time of challenge, six of these animals had no detectable virus in blood while the other four animals in the group presented virus titers of up to 10^2 to 10^3 HAD_{50}/ml. Altogether, the animals infected with ASFV-G-ΔMGF tended to present lower virus titers in blood but exhibited prolonged viremia relative to the animals inoculated with parental ASFV-G.

Animals inoculated with ASFV-G-ΔMGF virus are protected against challenge with virulent parental virus. In order to assess

TABLE 2 Survival and fever response following infection of swine via the i.m. route with different doses of ASFV-G-ΔMGF and parental ASFV-G

No. of 50% HAD and virus strain	No. of survivors/ total no. of animals	Mean time to death (no. of days ± SD)	Fever data		
			No. of days (± SD) to onset	No. of days (± SD) duration	Maximum daily temp (°F ± SD)
10^2					
ASFV-G	0/5	9.2 (0.45)	7.2 (0.84)	2 (0.71)	106.4 (0.67)
ASFV-G-ΔMGF	10/10	—[a]	—	—	102.2 (0.29)
10^4					
ASFV-G	0/5	7.4 (0.55)	3.6 (0.89)	3.8 (1.1)	106.9 (0.51)
ASFV-G-ΔMGF	10/10	—	—	—	102.7 (0.81)

[a] —, animals did not die or they did not present with fever during the observation period.

the effect of inoculation with ASFV-G-ΔMGF on the induction of protection against challenge with virulent parental virus, ASFV-G-ΔMGF-exposed animals were challenged with parental virulent ASFV-G. Two groups of pigs, those inoculated with 10^2 HAD_{50} of ASFV-G-ΔMGF and those inoculated with 10^4 HAD_{50} of ASFV-G-ΔMGF, were i.m. challenged at 28 dpi with 10^3 HAD_{50} of virulent parental ASFV-G. Animals were monitored daily for clinical signs and changes in body temperature.

Five naive animals that were challenged with parental ASFV-G using the same route and dose served as a control group. These animals displayed ASF-related signs by 5 days postchallenge (dpc), with the signs evolving into more-severe manifestations in the

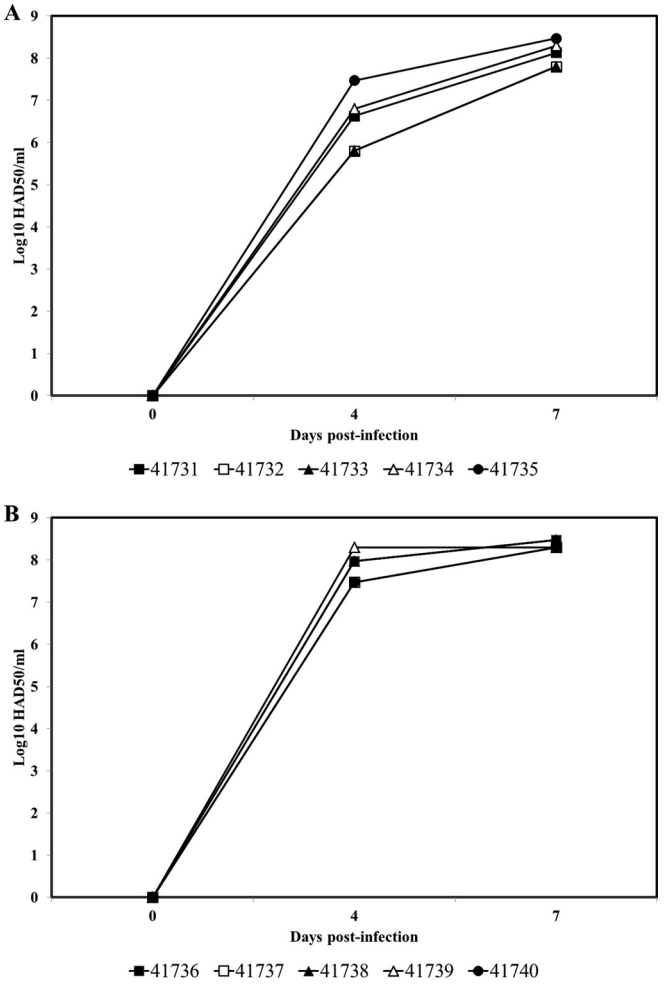

FIG 5 Virus titers in blood samples obtained from pigs infected i.m. with either 10^2 (A) or 10^4 (B) HAD_{50} of ASFV-G. Values are expressed as $\log_{10}$ HAD_{50}/ml. Sensitivity of virus detection, ≥$\log_{10}$ 1.8 HAD_{50}/ml.

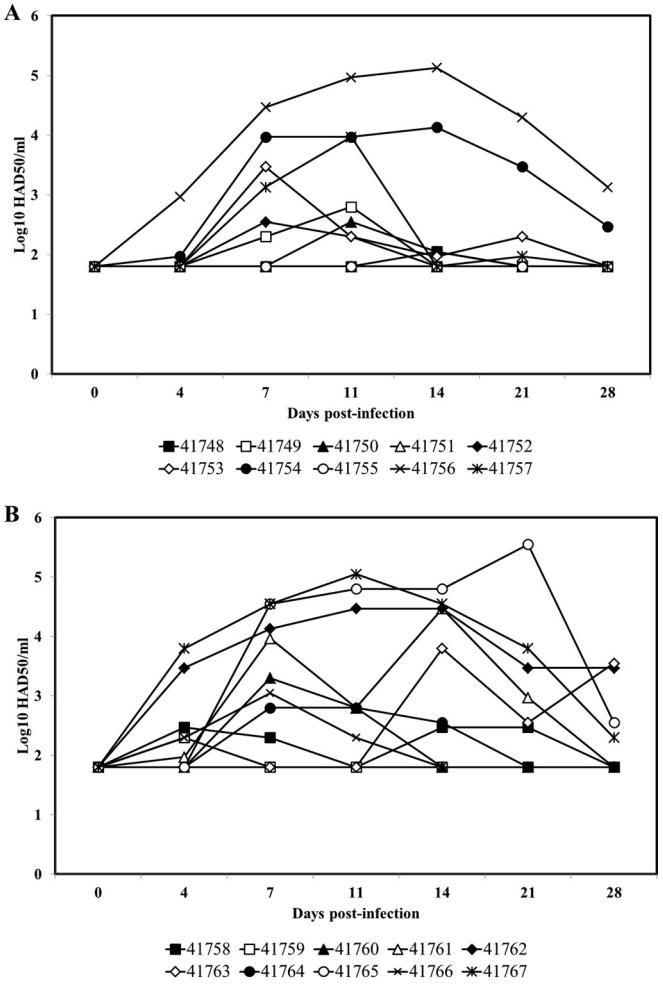

FIG 6 Virus titers in blood samples obtained from pigs infected i.m. with either 10^2 (A) or 10^4 (B) HAD_{50} of ASFV-G-ΔMGF. Values are expressed as $\log_{10}$ HAD_{50}/ml. Sensitivity of virus detection, ≥$\log_{10}$ 1.8 HAD_{50}/ml.

TABLE 3 Swine survival and fever response in animals infected via the i.m. route with either 10^2 or 10^4 HAD_{50} ASFV-G-ΔMGF and challenged at 28 dpi via the i.m. route with 10^3 HAD_{50} of parental ASFV-G[a]

Treatment	No. of survivors/ total no. of animals	Mean time to death (no. of days ± SD)	Fever data[b]		
			No. of days (± SD) to onset	No. of days (± SD) duration	Maximum daily temp (°F ± SD)
10^2 HAD_{50}	10/10	—[c]	7.5 (1.74)[d]	1 (0)	103.5 (1.04)
10^4 HAD_{50}	10/10	—	7.5 (4.42)[e]	1 (0)	103.7 (1.10)
Mock infection	0/5	8.2 (1.1)	5.20 (1.31)	3.0 (0.70)	106.5 (0.46)

[a] Data represent mean values and standard deviations.
[b] Rectal temperatures above 104°F.
[c] —, animals did not die or they did not present with fever during the observation period.
[d] Three of 10 animals presented with fever: 1 animal presented with 105.1°F at 4 dpi, and 2 animals presented with 104.2°F and 105.5°F at 7 dpi.
[e] Four of 10 animals presented with fever: 1 animal presented 105.8°F at 4 dpi, 2 animals presented with 104.8°F and 105.6°F at 6 dpi, and 1 animal presented with 104.2°F at 14 dpi.

following days and all animals dying or being euthanized around 8 dpc. All animals receiving either 10^2 or 10^4 HAD_{50} of ASFV-G-ΔMGF survived the challenge with the parental virulent virus. In addition, all of these pigs in general remained clinically normal during the observation period (21 days), with the exception that some of the animals presented a transitory moderate rise in body temperature lasting for a day (Table 3). Therefore, infection with either 10^2 or 10^4 HAD_{50} of ASFV-G-ΔMGF effectively induced protection against clinical disease and death upon challenge with parental virulent ASFV-G.

The presence of virus titers in blood observed upon challenge is an indicator of the protective potency of the immunity elicited by ASFV-G-ΔMGF. The presence of viremia postchallenge was detected (at different levels) in 8 of 10 animals in both groups (data not shown). The type of virus present in those blood samples was determined by conventional PCR, using primers specific for the β-GUS gene and for the MGF-1R gene as indicators of the presence of ASFV-G-ΔMGF and ASFV-G, respectively. The time points tested were selected based on the highest titer for each of the animals. Of the 10 animals in the group infected with 10^2 HAD_{50} of ASFV-G-ΔMGF, 1 did not show detectable viremia or positive PCRs. Another animal showed a very low titer that was not detected by the PCRs. Of the remaining eight animals, five were positive for ASFV-G-ΔMGF but were negative for ASFV-G, while the other three animals were positive for the presence of both viruses (Table 4). Regarding the animals infected with 10^4 HAD_{50} of ASFV-G-ΔMGF, six of them presented ASFV-G-ΔMGF in their blood samples but were negative for ASFV-G, while the other four animals were positive for both viruses (Table 4). Therefore, although complete protection against the clinical form of the disease was achieved, approximately 30% to 40% of the ASFV-G-ΔMGF-inoculated animals harbored the infection with the

TABLE 4 Viremia, virus detected in blood, antibody response, and fever in pigs exposed to ASFV-G-ΔMGF and challenged with parental ASFV-G

ASFV-G-ΔMGF HAD_{50}/time of challenge for indicated pig	Viremia (log_{10} HAD_{50}/ml)[a]	Virus detection in blood (PCR)		Anti-ASFV antibody titer (log_{10})[b]	Fever[c]
		ASFV-G-ΔMGF	ASFV-G		
10^2/28 dpi					
41748	2.05	+	+	3.81	No
41749	2.55	+	+	3.51	Yes
41750	4.55	+	+	2.9	Yes
41751	≤1.8	+	−	3.68	No
41752	≤1.8	+	−	3.81	No
41753	2.97	+	−	3.81	Yes
41754	2.47	+	−	3.81	No
41755	2.47	−	−	3.81	No
41756	2.47	+	−	3.81	No
41757	≤1.8	−	−	3.68	No
10^4/28 dpi					
41758	2.97	+	+	3.81	Yes
41759	6.12	+	+	3.68	Yes
41760	≤1.8	+	−	3.68	No
41761	≤1.8	+	−	3.81	No
41762	3.47	+	−	3.81	No
41763	≤1.8	+	−	3.68	No
41764	5.55	+	+	3.08	Yes
41765	3.3	+	−	3.81	No
41766	3.55	+	−	3.81	No
41767	4.55	+	+	3.81	Yes

[a] Viremia was assessed at 4 or 7 dpc; only the highest titers detected are shown. Sensitivity of detection, ≥log_{10}1.8 HAD_{50}/ml.
[b] Serum antibody titers (at 28 dpi) are expressed as log_{10} of the inverse of the highest dilution giving positive results.
[c] Animals showed a transient rise in rectal temperature above 104°F.

parental virus. Interestingly, most of the animals showing a transient rise in body temperature were those harboring challenge virus in their blood. Furthermore, regarding the presence of ASFV-G viremias or transient fever, all animals showed high levels of circulating anti-ASFV antibodies at the time of challenge.

DISCUSSION

Infection with live attenuated ASFV strains has been the only method available to effectively protect pigs against challenge with homologous virulent isolates. These attenuated viruses have been developed by sequential passages in cell cultures (8, 23–25) or by genetic manipulation. Attenuated viruses obtained by genetic manipulation involve the deletion of specific genes by a process of homologous recombination (9–12, 36). The generated recombinant deletion mutant viruses have significantly reduced virulence in swine, and, in all cases, animals inoculated with each of these genetically modified viruses survived the infection when challenged with the corresponding virulent parental virus (9–12, 36). Those findings suggest that development of attenuated ASFV recombinant viruses by genetic manipulations of a specific gene(s) is an effective approach for vaccine development.

The MGF360 and MGF505 genes exist in the highly variable left-terminal region of the ASFV genome and have been the focus of investigation since some naturally attenuated field isolates such as OURT 88/3 or NHV (37), or cell-culture adapted attenuated ASFV strains such as BA71v (15, 19–21) or ASFV-G/V110 (22), presented similar deletions in this region encompassing members of both MGFs (Fig. 2). For instance, ASFV-G/V110 lacks MGF360-13L and -14L along with MGF505-2R, -3R, -4R, and -5R; NHV and OURT 88/3 share exactly the same deletions affecting MGF360-10L, -11L, -12L, -13L, and -14L along with MGF505-1R and -2R; and BA71v has deletions encompassing MGF360-9L, -10L, -11L, -12L, -13L, and 14L along with MGF505-1R. Although all these attenuated viruses bear deletions in the left variable region of the genome encompassing MGF360 and MGF505 members, so far, a direct cause-effect relationship between attenuation of virulence and deletion of MGF genes has not been proven.

The role of MGF360 and MGF505 in determining the ASFV host range has been better studied. For instance, it has been shown that deletion of MGF360-12L, -13L, and -14L from the genome of the ASFV Pretoriuskop/96/4 isolate reduces the ability of the virus to replicate in ticks (28). More importantly, restoration of specific MGFs (MF360-9L, -10L, and -11L along with MGF505-1R or MF360-12L, -13L, and -14L) in Vero cell-adapted BA71v partially restores the ability of the virus to replicate in swine macrophages (27). Conversely, deletion of MF360-9L, -10L, -11L, -12L, -13L, and -14L along with MGF505-1R and -2R genes significantly reduces the ability of the ASFV Pretoriuskop/96/4 isolate to replicate in swine macrophages (27). In addition, this deletion abrogates the ability of Pretoriuskop/96/4 virus to inhibit the type I IFN expression in infected swine macrophages that is otherwise observed in infections with the parental virus (29).

The ASFV *NL* (*DP71L*) gene product exists in two different forms, a long form (184 amino acids) or a short form (70 to 72 amino acids), depending on the ASFV isolate (11). Deletion of this gene in the ASFV E70 isolate (short form) rendered an attenuated virus, whereas deletion of the *NL* (*DP71L*) gene from ASFV Malawi Lil-20/1 (long form) or Pretoriuskop/96/4 (short form) did not result in attenuation of the viruses (30). Interestingly, when the genome of the Malawi Lil-20/1-ΔNL virulent recombinant virus (30) was genetically modified to introduce a deletion of MGF360 and MGF505 genes similar to the deletion introduced in ASFV-G that produced ASFV-G-ΔMGF, the resulting virus showed an attenuated phenotype in swine (31). As stated by those authors, the deletion of these MGF genes from Malawi Lil-20/1-ΔNL complemented the *NL* gene function. From that work, it is not possible to discern if the deletion of MGF360 and MGF505 genes from the virulent Malawi Lil-20/1 isolate would result in virus attenuation. In addition, the ASFV Malawi Lil-20/1 isolate lacking the *NL* gene and MGF genes reported by Neilan et al. (31) was not tested as an experimental vaccine to assess the protective efficacy induced by the recombinant virus against challenge with virulent ASFV.

As a summary, we report here the first evidence that deletion of members of MGF360 and MGF505 is able to confer attenuation to a highly virulent ASFV isolate. In addition, the ASFV-G-ΔMGF data constitute the only experimental evidence so far of immunogens able to induce protection against challenge with the highly virulent ASFV-G isolate. Certainly, the use of genetically modified viruses as live attenuated vaccines may represent a safety risk. For instance, the long-term effects of such a development of chronic disease as a consequence of infecting animals with recombinant attenuated ASFVs need to be assessed. Additionally, the long-term response of animals which were protected against challenge with virulent virus also needs to be addressed. Here we have observed that a proportion of challenged animals, although protected, harbored the wild-type virulent virus. The long-term effect of this condition is not known.

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REFERENCES

1. **Costard S, Wieland B, de Glanville W, Jori F, Rowlands R, Vosloo W, Roger F, Pfeiffer DU, Dixon LK.** 2009. African swine fever: how can global spread be prevented? Philos Trans R Soc Lond B Biol Sci **364:**2683–2696. http://dx.doi.org/10.1098/rstb.2009.0098.
2. **Tulman ER, Delhon GA, Ku BK, Rock DL.** 2009. African swine fever virus. Curr Top Microbiol Immunol **328:**43–87.
3. **Chapman DA, Darby AC, Da Silva M, Upton C, Radford AD, Dixon LK.** 2011. Genomic analysis of highly virulent Georgia 2007/1 isolate of African swine fever virus. Emerg Infect Dis **17:**599–605. http://dx.doi.org/10.3201/eid1704.101283.
4. **Coggins L.** 1974. African swine fever virus. Pathogenesis. Prog Med Virol **18:**48–63.
5. **Kihm U, Ackerman M, Mueller H, Pool R.** 1987. Approaches to vaccination, p 127–144. *In* Becker Y (ed), African swine fever. Martinus Nijhoff Publishing, Boston, MA.
6. **Mebus CA.** 1988. African swine fever. Adv Virus Res **35:**251–269. http://dx.doi.org/10.1016/S0065-3527(08)60714-9.
7. **Hamdy FM, Dardiri AH.** 1984. Clinical and immunologic responses of pigs to African swine fever virus isolated from the Western Hemisphere. Am J Vet Res **45:**711–714.
8. **Ruiz Gonzalvo F, Caballero C, Martinez J, Carnero ME.** 1986. Neutralization of African swine fever virus by sera from African swine fever-resistant pigs. Am J Vet Res **47:**1858–1862.
9. **Moore DM, Zsak L, Neilan JG, Lu Z, Rock DL.** 1998. The African swine

fever virus thymidine kinase gene is required for efficient replication in swine macrophages and for virulence in swine. J Virol **72:**10310–10315.
10. **Lewis T, Zsak L, Burrage TG, Lu Z, Kutish GF, Neilan JG, Rock DL.** 2000. An African swine fever virus ERV1-ALR homologue, 9GL, affects virion maturation and viral growth in macrophages and viral virulence in swine. J Virol **74:**1275–1285. http://dx.doi.org/10.1128/JVI.74.3.1275-1285.2000.
11. **Zsak L, Lu Z, Kutish GF, Neilan JG, Rock DL.** 1996. An African swine fever virus virulence-associated gene NL-S with similarity to the herpes simplex virus ICP34.5 gene. J Virol **70:**8865–8871.
12. **Zsak L, Caler E, Lu Z, Kutish GF, Neilan JG, Rock DL.** 1998. A nonessential African swine fever virus gene UK is a significant virulence determinant in domestic swine. J Virol **72:**1028–1035.
13. **Almendral JM, Almazan F, Blasco R, Vinuela E.** 1990. Multigene families in African swine fever virus: family 110. J Virol **64:**2064–2072.
14. **González A, Calvo V, Almazan F, Almendral JM, Ramirez JC, de la Vega I, Blasco R, Vinuela E.** 1990. Multigene families in African swine fever virus: family 360. J Virol **64:**2073–2081.
15. **de la Vega I, Vinuela E, Blasco R.** 1990. Genetic variation and multigene families in African swine fever virus. Virology **179:**234–246. http://dx.doi.org/10.1016/0042-6822(90)90293-Z.
16. **Rodriguez F, Alcaraz C, Eiras A, Yanez RJ, Rodriguez JM, Alonso C, Rodriguez JF, Escribano JM.** 1994. Characterization and molecular basis of heterogeneity of the African swine fever virus envelope protein p54. J Virol **68:**7244–7252.
17. **Vydelingum S, Baylis SA, Bristow C, Smith GL, Dixon LK.** 1993. Duplicated genes within the variable right end of the genome of a pathogenic isolate of African swine fever virus. J Gen Virol **74**(Pt 10)**:**2125–2130.
18. **Yozawa T, Kutish GF, Afonso CL, Lu Z, Rock DL.** 1994. Two novel multigene families, 530 and 300, in the terminal variable regions of African swine fever virus genome. Virology **202:**997–1002. http://dx.doi.org/10.1006/viro.1994.1426.
19. **Pires S, Ribeiro G, Costa JV.** 1997. Sequence and organization of the left multigene family 110 region of the Vero-adapted L60V strain of African swine fever virus. Virus Genes **15:**271–274. http://dx.doi.org/10.1023/A:1007992806818.
20. **Tabares E.** 1987. Characterization of ASF virus proteins, p 51–62. *In* Becker Y. (ed) African swine fever. Martinus Nijhoff, Boston, MA.
21. **Yáñez RJ, Rodriguez JM, Nogal ML, Yuste L, Enriquez C, Rodriguez JF, Vinuela E.** 1995. Analysis of the complete nucleotide sequence of African swine fever virus. Virology **208:**249–278. http://dx.doi.org/10.1006/viro.1995.1149.
22. **Krug PW, Holinka LG, O'Donnell V, Reese B, Sanford B, Fernandez-Sainz I, Gladue DP, Arzt J, Rodriguez L, Risatti GR, Borca MV.** 2015. The progressive adaptation of a Georgian isolate of African swine fever virus to Vero cells leads to a gradual attenuation of virulence in swine corresponding to major modifications of the viral genome. J Virol **89:**2324–2332. http://dx.doi.org/10.1128/JVI.03250-14.
23. **Boinas FS, Hutchings GH, Dixon LK, Wilkinson PJ.** 2004. Characterization of pathogenic and non-pathogenic African swine fever virus isolates from Ornithodoros erraticus inhabiting pig premises in Portugal. J Gen Virol **85:**2177–2187. http://dx.doi.org/10.1099/vir.0.80058-0.
24. **Enjuanes L, Carrascosa AL, Moreno MA, Vinuela E.** 1976. Titration of African swine fever (ASF) virus. J Gen Virol **32:**471–477. http://dx.doi.org/10.1099/0022-1317-32-3-471.
25. **Martins CL, Lawman MJ, Scholl T, Mebus CA, Lunney JK.** 1993. African swine fever virus specific porcine cytotoxic T cell activity. Arch Virol **129:**211–225. http://dx.doi.org/10.1007/BF01316896.
26. **Zsak L, Lu Z, Burrage TG, Neilan JG, Kutish GF, Moore DM, Rock DL.** 2001. African swine fever virus multigene family 360 and 530 genes are novel macrophage host range determinants. J Virol **75:**3066–3076. http://dx.doi.org/10.1128/JVI.75.7.3066-3076.2001.
27. **Zsak L, Neilan JG.** 2002. Regulation of apoptosis in African swine fever virus-infected macrophages. ScientificWorldJournal **2:**1186–1195. http://dx.doi.org/10.1100/tsw.2002.214.
28. **Burrage TG, Lu Z, Neilan JG, Rock DL, Zsak L.** 2004. African swine fever virus multigene family 360 genes affect virus replication and generalization of infection in Ornithodoros porcinus ticks. J Virol **78:**2445–2453. http://dx.doi.org/10.1128/JVI.78.5.2445-2453.2004.
29. **Afonso CL, Piccone ME, Zaffuto KM, Neilan J, Kutish GF, Lu Z, Balinsky CA, Gibb TR, Bean TJ, Zsak L, Rock DL.** 2004. African swine fever virus multigene family 360 and 530 genes affect host interferon response. J Virol **78:**1858–1864. http://dx.doi.org/10.1128/JVI.78.4.1858-1864.2004.
30. **Afonso CL, Zsak L, Carrillo C, Borca MV, Rock DL.** 1998. African swine fever virus NL gene is not required for virus virulence. J Gen Virol **79**(Pt 10)**:**2543–2547.
31. **Neilan JG, Zsak L, Lu Z, Kutish GF, Afonso CL, Rock DL.** 2002. Novel swine virulence determinant in the left variable region of the African swine fever virus genome. J Virol **76:**3095–3104. http://dx.doi.org/10.1128/JVI.76.7.3095-3104.2002.
32. **Reed LJ, Muench HA.** 1938. A simple method of estimating fifty per cent endpoints. Am J Hyg **27:**493–497.
33. **Neilan JG, Lu Z, Kutish GF, Zsak L, Lewis TL, Rock DL.** 1997. A conserved African swine fever virus IkappaB homolog, 5EL, is nonessential for growth in vitro and virulence in domestic swine. Virology **235:**377–385. http://dx.doi.org/10.1006/viro.1997.8693.
34. **Sanger F, Nicklen S, Coulson AR.** 1977. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A **74:**5463–5467. http://dx.doi.org/10.1073/pnas.74.12.5463.
35. **Huang X, Madan A.** 1999. CAP3: a DNA sequence assembly program. Genome Res **9:**868–877. http://dx.doi.org/10.1101/gr.9.9.868.
36. **Neilan JG, Zsak L, Lu Z, Burrage TG, Kutish GF, Rock DL.** 2004. Neutralizing antibodies to African swine fever virus proteins p30, p54, and p72 are not sufficient for antibody-mediated protection. Virology **319:**337–342. http://dx.doi.org/10.1016/j.virol.2003.11.011.
37. **Chapman DA, Tcherepanov V, Upton C, Dixon LK.** 2008. Comparison of the genome sequences of non-pathogenic and pathogenic African swine fever virus isolates. J Gen Virol **89:**397–408. http://dx.doi.org/10.1099/vir.0.83343-0.